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TABLE OF CONTENTS

INTRODUCTION

The major failure in breast cancer management is the incomplete killing of malignant tumour cells that have spread throughout the body [1]. This is despite the many treatments available, such as surgery, radiation therapy, hormone therapy and chemotherapy. The American Cancer Society estimated 182,800 new cases of invasive breast cancer in the year 2000 among women in America, and 40,800 are expected to die from the disease [2]. Novel, more effective treatments that overcome this problem in breast cancer management are essential. Targeted therapy, first discussed over 100 years ago, is based on the idea that a drug will attack its target without damaging other tissue [3]. Targeted alpha therapy (TAT) uses an alpha emitting radionuclide as a lethal medicament via an effective targeting carrier to kill cancer cells [5]. We are investigating a novel TAT approach that exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells.

Alpha emitting radionuclides emit alpha particles with energies up to an order of magnitude greater than most beta rays, yet their ranges are two orders of magnitude less as alpha particles have a linear energy transfer (LET) which is about 100 times greater [5]. This is manifested by a high relative biological effectiveness (RBE). As a result, a much greater fraction of the total energy is deposited in cells with alphas and very few nuclear hits are required to kill a cell. Consequently, 100-fold enhancement in radiation dose [6, 7] would be delivered to the nucleus of a cancer cell if an effective carrier were employed to take the alpha radionuclide into that cancer cell. Thus, only alpha radiation has the potential to kill the metastatic cancer cells at tolerable dose limits, whereas the low LET of betas make this a very difficult task within human dose tolerance limits.

Availability of the alpha emitting radionuclides has been the major problem in the past for their large-scale scientific and clinical application. Studies have been carried out using ^{149}Tb [5,21], ^{211}At and ^{212}Bi [8-11] with encouraging results. The stable and reliable ^{225}Ac generator of the alpha emitting nuclide ^{213}Bi has been produced, modified and used successfully [11-22], with several of these studies indicating a therapeutic potential of ^{213}Bi-labeled antibody constructs against cancer cells both in vitro and in vivo. Our group has modified methods of conjugating ^{213}Bi radionuclide to antibodies with the stable chelator cyclic diethylenetriaminepentacetic acid anhydride (cDTPAa) for use in the alpha therapy of melanoma [18], colorectal cancer [21], leukaemia [22] and prostate cancer [48] and breast cancer [49].

A large body of experimental and clinical evidence implicates over-expression of the urokinase plasminogen activator (uPA) system as a modulator of the aggressive behaviour of tumour cells and as a strong prognostic factor for predicting poor breast cancer patient outcome [23-25]. uPA converts plasminogen into the highly active protease plasmin [23]. Plasmin promotes tissue degradation and remodelling of the local extracellular environment by directly and indirectly (via activation of pro-metalloproteases) degrading extracellular matrix molecules [23-25]. uPA is synthesised and secreted as a pro-enzyme, whose activation is markedly accelerated upon binding with high affinity (0.1 – 1 nM) to specific cell surface uPA receptors (uPAR) [23,26]. Receptor density varies depending on cell type (10^3 - 10^6 sites/cell) [26].

The ability of PAI-2 to inhibit tumour invasion and metastases in animal models has been demonstrated by several laboratories utilising uPA-overexpressing cancer cells. For example, local invasion of human sarcoma xenografts is limited by stable expression of PAI-2 in the sarcoma cells [42], over-expression of PAI-2 in human melanoma cells inhibits spontaneous metastasis in immuno-compromised mice [43], and pre-treatment of rat mammary cancer cells with recombinant human PAI-2 or slow infusion of the inhibitor with osmotic pumps led to a significant decrease in lung metastasis post intravenous administration [44]. Since ^{125}I-PAI-2 was shown to accumulate in uPA-

overexpressing colorectal cancer cell xenografts in mice [36], and PAI-2 levels were shown to be negligible in invasive colorectal cancer tissues that contained high levels of uPA antigen [45], it is likely that exogenous PAI-2 can target invasive tumours in a uPA-dependent manner. Moreover, since localisation studies indicate that quiescent, normal or benign tissues do not contain significant levels of uPA (refer to Introduction) it is unlikely that uPA-targeted therapy will appreciably affect normal tissues.

PAI-1 conjugated to A-chain cholera toxin as the cytotoxic agent or modified PAI-1 conjugated to saporin has been used to target fibrosarcoma cells [46,47] with moderate cytotoxicity. However, PAI-2 has several distinct advantages over PAI-1 for targeted cancer therapy. Firstly, PAI-2 is 10,000 fold less active than PAI-1 towards tissue type plasminogen activator, the latter having a high affinity for fibrin, indicating that administered PAI-2 would not adversely affect fibrinolysis and hemostasis [35]. PAI-2 is very stable in vitro compared to PAI-1 and does not revert to a latent form in vitro or in vivo compared to PAI-1 [35]. Sustained exposures to PAI-2 are unlikely to cause adverse health effects since the "abnormally" high PAI-2 levels found during late pregnancy (usually blood levels of PAI-2 are undetectable) are not associated with toxicity [35]. In addition, obstacles associated with targeted immunotherapy, such as large protein size and human anti-mouse antibody responses, both of which require significant antibody manipulation to overcome such problems, are not a concern with PAI-2.

Immunohistochemistry mirrored the endogenous uPA and uPAR antigen expression differences seen in two breast cancer cell lines, ie MDA-MB-231 and MCF7, by flow cytometry. Notably, the staining patterns of both antigens for both cell lines were punctuate and heterogeneous [49]. Most of the MDA-MB-231 cells were highly positive for either antigen with less than 10% being weakly positive. In contrast, most of the MCF-7 cells were only weakly positive with less than 10% being moderately positive.

Cell surface-bound uPA is accessible to and inhibitable by exogenous PAI2 [35,36], and a number of studies have suggested the potential for PAI-2 to inhibit cancer cell invasion and metastasis [35].

The pharmacokinetics and biodistribution of human recombinant ^{125}I-labelled PAI-2 in both control mice and mice bearing human colon cancer (uPA-positive HCT116 cell line) xenografts has been established [36]. Briefly, ^{125}I-PAI2 localised in 0.5 cm^3 tumour xenografts quickly (after 1 min, peaking at 30 - 60 min at approx. 1.5% of total injected dose). Furthermore, repeat intravenous, subcutaneous, or intra-peritoneal injections of ^{125}I-PAI2 resulted in an accumulation of radioactivity without an accompanying increase in the major organs or in toxicity. In addition, tumour associated ^{125}I-PAI2 correlated with tumour mass. Such studies indicate that invasive and metastatic tumour cells, shown consistently to contain active uPA, would be accessible to and targeted by exogenously administered PAI2.

It is clear that uPA is a specific marker of malignancy and that PAI2 represents a useful targeting agent. We have previously reported the production and evaluation of the new alpha-nuclide emitting cytotoxic agent ^{213}Bi-labeled PAI2 (alpha-PAI2 or API) [49]. The reactivity and specificity of alpha-PAI2 cytotoxicity was reported for two human breast cancer cell lines in vitro. We now demonstrate the efficacy of TAT with API in inhibiting the growth of tumours in vivo, within dose tolerance limits. These data clearly show that alpha-PAI2 has an important role as a potential new therapeutic modality for the control of micrometastases in breast cancer.

MATERIALS AND METHODS

Materials

Human recombinant PAI-2 (47 kDa) was provided by Biotech Australia Pty Ltd. Glu-plasminogen was purified from human plasma as described by Andronicos et al [37]. Microspin concentrators were purchased from Millipore (Bedford, MA, USA). RPMI-1640 was purchased from Life Technologies (Castle Hill, NSW, Australia). Fetal calf serum (FCS) was obtained from Trace Bioscientific (Castle Hill, NSW, Australia). The cyclic anhydride of diethylenetriaminepentacetic acid (cDTPA) was purchased from Aldrich Chemical Company. Bovine serum albumin (fraction V) (BSA) and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO. USA). Human twin chain urokinase plasminogen activator (tc-uPA) was purchased from Serono (Sydney, NSW, Australia). Glu-gly-arg chloromethylketone (EGR-CMK) was purchased from Calbiochem (Sydney, NSW, Australia). Spectrozyme-UK (carobenzoxy-L-γ-glutamyl-(α-t-butoxy)-glycyl-arginine-p-nitroanilide-diacetate), mouse anti-human uPA IgG_1 (#394), mouse anti-human uPAR IgG_{2a} (#3696), and mouse anti-human PAI-2 IgG_1 (#3750) monoclonal antibodies were purchased from American Diagnostica Inc (Greenwich, CT, USA). Mouse isotype control subclasses IgG_1, IgG_{2a} antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG were from Silenus (Sydney, NSW, Australia). Fresh human leukocytes were isolated from whole blood using Fycoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions.

Cell lines

MDA-MB-231 breast adenocarcinoma, pleural effusion, human.
MCF-7 originally isolated from breast adenocarcinoma, pleural effusion, human.
MCF-7 cells, unlike MDA cells, retain several characteristics of differentiated mammary epithelial cells.

Radioisotope

Alpha particle emitting radionuclide, ^{213}Bi, was eluted from the $^{225}Ac/^{213}Bi$ generator which was purchased from the Oak Ridge National Laboratory, United States Department of Energy. ^{213}Bi was eluted from the ^{225}Ac column with 250 µL of freshly prepared 0.15 M distilled and stabilized hydriodic acid followed by washing with 250 µL sterile distilled water [38]. The first elution was not used, and a time of 2 h was allowed for ^{213}Bi to regenerate on the column for the next elution. Corrections were made for ^{213}Bi decay using the half-life of 46 min f or all activity calculations.

PAI-2 conjugation with cDTPA, stoichiometry and reactivity

PAI-2 and BSA were conjugated with cDTPA by a modification of the method described by Boll et al. [38] and Paik et al [39], to give the desired protein-DTTA conjugate. PAI-2/BSA (1 mg) dissolved in PBS was conjugated to cDTPA by first increasing the pH to approximately 8.2 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). A 50 fold molar excess of cDTPA (found to result in smallest fraction of underivatised PAI-2; data not shown) was added and the mixture incubated at 25°C for 1 h with intermittent rocking. The reaction was stopped with a final concentration of 10% (v/v) 1 M Tris-HCl (pH7.2). The final reaction volume was 0.5 mL Three reaction volumes of PBS were used to purify DTTA-labeled proteins away from free cDTPA using a microspin concentrator as described by the manufacturer.

The concentrations of the protein-DTTA conjugates were measured by BIORAD DC protein assay reagent kit. The stoichiometry of DTTA-PAI-2 was determined using electrospray ionisation mass spectrometry as previously described [49].

^{213}Bi labeling of DTTA-PAI-2

Concentrated DTTA-PAI-2 stocks were diluted with 500 mM sodium acetate at pH 5.5 and 5 – 10 • g of DTTA-PAI-2 was labeled with free ^{213}Bi for 20 min at room temperature. After labeling, ^{213}Bi-DTTA-PAI-2 (alpha-PAI-2) was buffer exchanged into PBS using a PD-10 column using PBS (pH 7.0) as the eluting buffer. ^{213}Bi-DTTA-BSA (alpha-BSA) was radiolabeled by similar methods.

radioisotope in each section was counted using a 340-540 keV window. The radiolabeled protein is found at the origin section, while free radioisotope is found at the solvent front section.

Cell culture

The metastatic MDA-MB-231 human breast cancer cell line was originally isolated from human breast adenocarcinoma (pleural effusion) and does not carry characteristics of differentiated mammary epithelial cells [41]. Cells were routinely cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS and passaged using Trypsin/EDTA. The cells were incubated in a humidified incubator at 37 °C with a 5% carbon dioxide air atmosphere. For all experimental procedures, sub-confluent cells that had been in culture for 48 h without a change of media were harvested by rinsing flasks twice with PBS (pH 7.2) and then detaching with PBS/0.5mM EDTA at 37 °C for 5 min. Cells were collected and resuspended in the appropriate buffer as described below.

Flow cytometry

For the detection of cell-surface uPA, uPAR and PAI2 indirect immunofluorescence staining was performed as described by Ranson et al. [41]. All flow cytometry data were analysed using CELLQuest software (Becton-Dickinson). Isotype control fluorescences were subtracted from all flow cytometry experiments.

Immunohistochemistry

Cells grown on glass coverslips for 48 h in growth medium, were washed three times with PBS at room temperature, fixed by incubation with PBS supplemented with 1% gluteraldehyde for 1 h at room temperature, and then washed once with PBS. Endogenous peroxidases were inactivated with 3% H_2O_2 for 5 min at room temperature. The cells were incubated with 10% human serum for 30 min, washed with PBS, and then incubated for 1 h with primary antibodies diluted with PBS/10 % human serum (final concentration 20 µg/mL) at room temperature. The cells were washed twice with PBS and incubated with the secondary reagents from the DAKO LSAB+ Kit according to the manufacturer's instructions. After rinsing with PBS, the cells were viewed using a video camera (National Panasonic) attached to an inverted compound microscope (Leica, Germany). Images of the cells (original magnification x400 unless otherwise stated) were captured by a Power PC (Macintosh 8500/20) using Apple Video Player software (Macintosh).

Cytotoxicity assay

The CellTiter 96 Aqueous non-radioactive cell proliferation assay (Promega, WI, USA) was used to determine the effect of ^{213}Bi labeled proteins on cell survival.

The activities of alpha-PAI2 and alpha-BSA preparations were measured using a radioisotope calibrator and neutralized to pH 7.0 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). Immediately after this, five serial doses of alpha-PAI2 and one dose of alpha-BSA were prepared in 100 µL RPMI/10%FCS and added to 96-well plates in triplicate containing 20,000 cells/well in 100 µL RPMI medium/10% FCS. The plates were then incubated overnight in a 5% carbon dioxide atmosphere at 37 °C. Controls were performed in triplicate in the same 96-well plate for each experiment and consisted of RPMI/10% FCS medium alone. In all cases the cells had been previously pre-incubated with 20 µg/mL plasminogen for 20 min at room temperature and washed before placing into 96-well plates. In some cases, the cells were also treated with 0.5 mM EGR-CMK (a specific uPA inhibitor) [36] for 15 min at room temperature after plasminogen activation and prior to incubation with radiolabeled proteins.

The cells were then washed and incubated with 100 µL phenol-red free RPMI without FCS containing 20 µL of the CellTiter 96 reagent. After 3 h incubation in a 5% carbon dioxide atmosphere at 37 °C, the reaction was stopped by the addition of 10% SDS, and the absorbance in each well was recorded at 490 nm using a SPECTROmax plate reader. The absorbance reflects the number of surviving cells. Blanks were subtracted from all data and analysed using Prism software (GraphPad Software Inc, USA).

In vivo toxicity

6 weeks old Arc(s) nu/nu female mice were purchased from Animal Resources Centre, Western Australia. Groups of 3 mice received 1.5, 3 and 6 mCi/kg weight dose of alpha-PAI2 by i.p. injection, other mice were treated with PAI2, cDTPAa, and saline as controls.

In vivo efficacy

Six to eight week old Arc(s) nu/nu female mice received a sc inoculation of $2x10^6$ cells of the human breast cancer cell line MDA-MB-231 in serum free medium into bilateral mammary fat pads of each nude mouse. All mice were sacrificed when the xenografted tumours approached 1 cm^2 in diameter. Efficacy studies of local TAT were made for dose response at 2 days post-inoculation, and for post-inoculation time response.

Mice were classified into 4 groups for 4 different therapy time points: 2-4 days (5+5 mice), 7 days (5 mice), 14 days (5 mice), and 28 days (5 mice) after cell inoculation. Each group had 1 mouse as control and 4 mice as treated mice.

The dose response for systemic administration (ip injection at 2 days post-inoculation) was also studied.

RESULTS

Cytotoxicity of alpha-PAI2 towards the MDA-MB-231 cell lines

Alpha-PAI2 was found to be highly toxic to MDA-MB-231 (Figure 1). In contrast, alpha-BSA showed only slight toxicity compared with alpha-PAI2 at the maximum activity used. No significant toxicity was observed with either DTTA-PAI2 or PAI2. The D_o (37% cell survival) values with alpha-PAI2 were calculated to be **2.1 ± 0.2** μCi (n = 6) for the MDA-MB-231 cell line.

Tolerance study

The weights of treated mice reduced initially by 5-10%, than recovered after 1 week. Mice were monitored and weight measured. After 13 weeks, the saline control mouse died, but other mice lived very well until euthanasia at 24 weeks post-therapy. Results for the 6 mCi/kg group are shown in Fig 2.

Tumour model

Xenografted tumours were induced successfully at 28/32 cell inoculation sites, first tumours becoming visible at around 2-3 weeks post-inoculation. Tumour area of 1 cm^2 is reached after 4-6 weeks, all mice were sacrificed before 6 weeks. Histopathological analysis confirmed breast cancer cells in the tumours and metastatic cancer cells were found in the lymph nodes. Thus, a human breast cancer model, including a lymphatic pathway metastasis model, was established.

Local TAT

Local injection at 2 days of 12, 25 and 50 μCi of alpha-PAI2 per mouse showed a dose dependent response in groups of 5 mice. Control tumours grew quickly, while the 12 μCi group grew very slowly. The 25 and 50 μCi groups showed complete inhibition of tumour growth (Fig 3).

A single injection of alpha-PAI2 (25 μCi) was made into cell inoculation sites or tumours. Mice were monitored and tumours were measured.

The 2-4 day group had the best response to the therapy, which had around 50% (23/40) tumour control and slower tumour growth rate compared with control mice.

7 day and 14 day groups had 2/8 and 1/8 tumour control and slower growth rate compared with control mice.

The 28 day group had 0/8 tumour control and no obvious change in tumour growth rate.

Systemic TAT

Mice received ip injections of 1.5, 3 and 6 mCi/kg alpha-PAI2 at 2 days post inoculation. Results are shown in Fig 4 and indicate a dose response effect.

Systemic TAT

Mice received ip injections of 1.5, 3 and 6 mCi/kg alpha-PAI2 at 2 days post inoculation. Results are shown in Fig 4 and indicate a dose response effect.

DISCUSSION

A number of novel techniques that target the uPA system for tumour therapy have been suggested and are being investigated [25]. We have investigated the use of PAI-2 as the basis for a new therapeutic agent. In this study we describe for the first time the novel compound alpha-PAI-2 and show that it retains reactivity and selectivity towards uPA expressing breast cancer cells in vitro.

That alpha-PAI2 cytotoxicity is significantly mediated via a uPA-dependent mechanism was further confirmed by the lack of cytotoxicity of freshly isolated normal human leukocytes on which cell-surface localised active uPA was not detectable [Ranson et al 2001]. Furthermore, breast cancer cells incubated with the non-specific alpha-BSA were also minimally affected. Clearly, alpha-PAI2 is very toxic to targeted cancer cells, whereas non-targeted cells are spared from the radiotoxicity arising from the alpha radiation. These results underscore the potential usefulness of alpha-PAI2 in vivo.

The in vivo studies revealed that alpha-PAI2 can target isolated cells and preangiogenic cell clusters, as is the situation at 2 days post-inoculation of breast cancer cells. Local therapy required only 25 μCi to achieve complete inhibition of tumour genesis. A much higher dose of 100μCi was required for systemic administration to achieve 80% control. Alpha-PAI2 is increasingly less effective in a single dose protocol as the tumours grow in size. Clearly, the potential role of alpha-PAI2 lies in its ability to target and kill the most malignant cells that participate in the generation of micrometastases.

CONCLUSIONS

We have combined the cytotoxicity of an alpha-emitting radioisotope (^{213}Bi) with the targeting potential of PAI-2 towards the uPA system to create a novel construct alpha-PAI-2, a potential new therapeutic for targeted alpha therapy of cancer. The in vitro cytotoxicity of alpha-PAI-2 on breast cancer cells was shown to be specific by several means indicating that the cell killing ability of alpha-PAI-2 depends critically on the targeting of cells in a receptor bound, active uPA-dependent manner. The in vivo results show conclusively that alpha-API2 can target and kill isolated cells and cell clusters, and as such, is indicated for the control of micrometastic breast cancer.

FIGURES

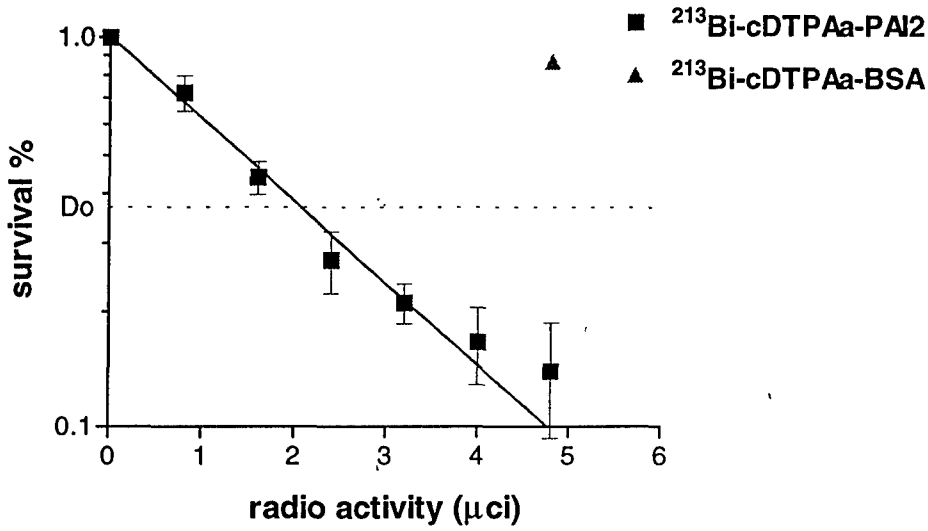

Figure 1. Cytotoxicity study of MDA-MB-231 cells treated with varying concentrations of alpha-PAI2 or a single concentration of alpha-BSA (•), incubated overnight and cell survival measured and expressed as a percentage of cell survival of control cells. Controls consisted of RPMI medium alone. Values shown are the means of two experiments performed in triplicate.

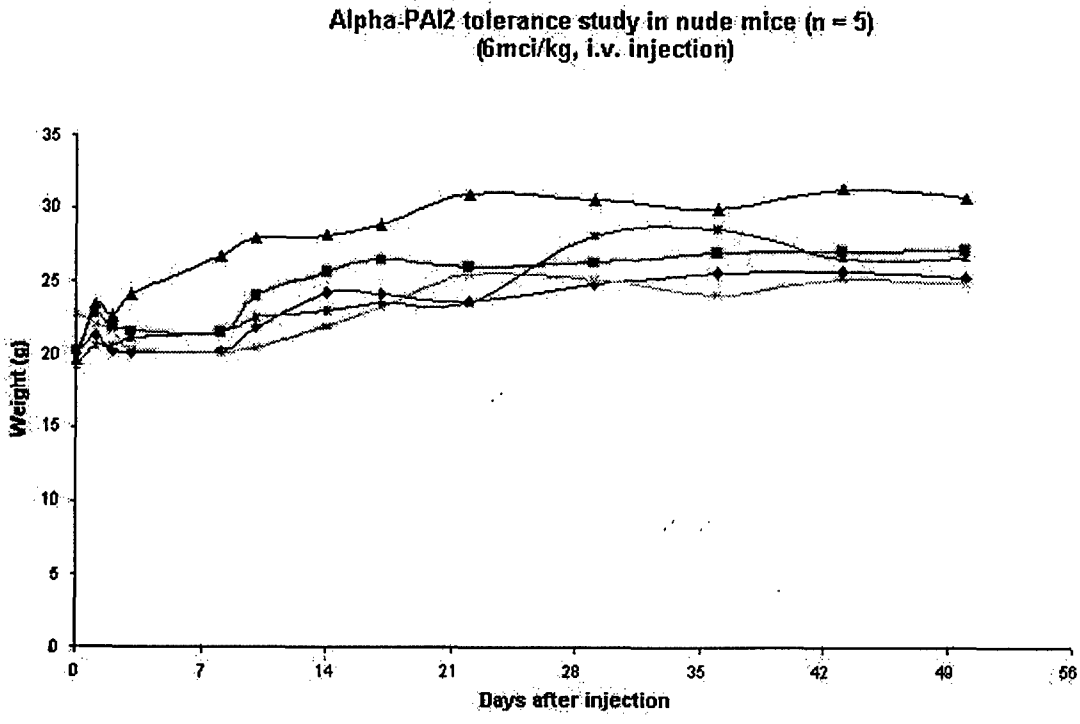

Figure 2. Toxicity studies at 1.5, 3 and 6 mCi/kg show no long term weight loss in nude mice after ip injection.

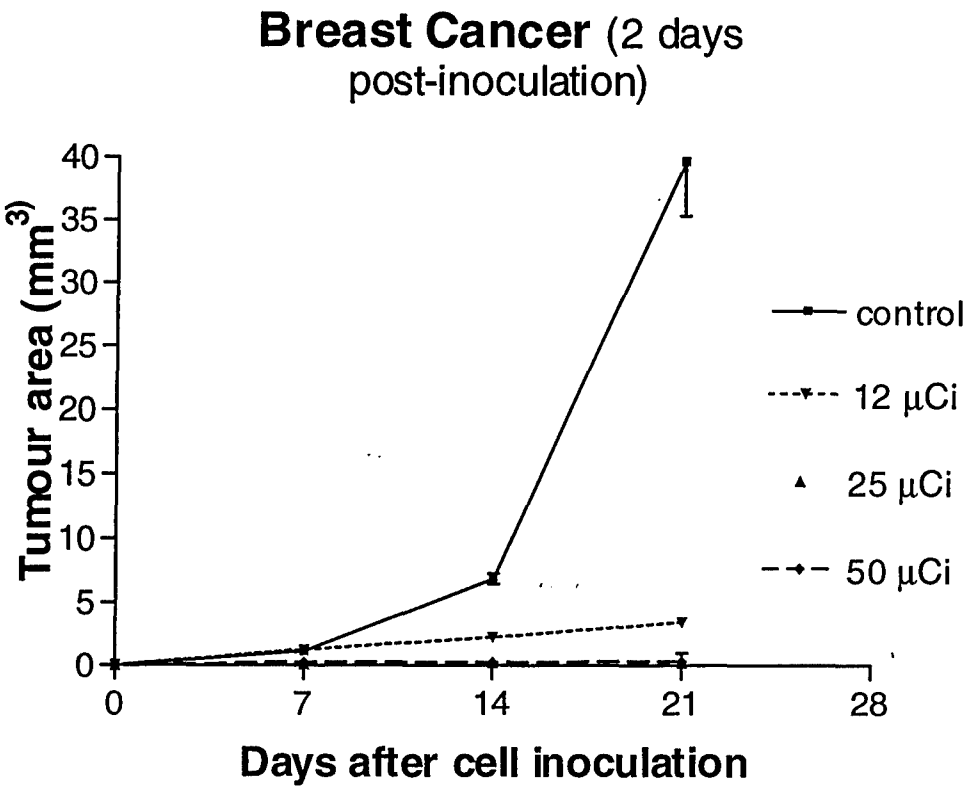

Figure 3. Inhibition of tumour growth by local injection of alpha-PAI2 at 2 days post-inoculation of $2x10^6$ MDA cells. An indicative dose response is apparent.

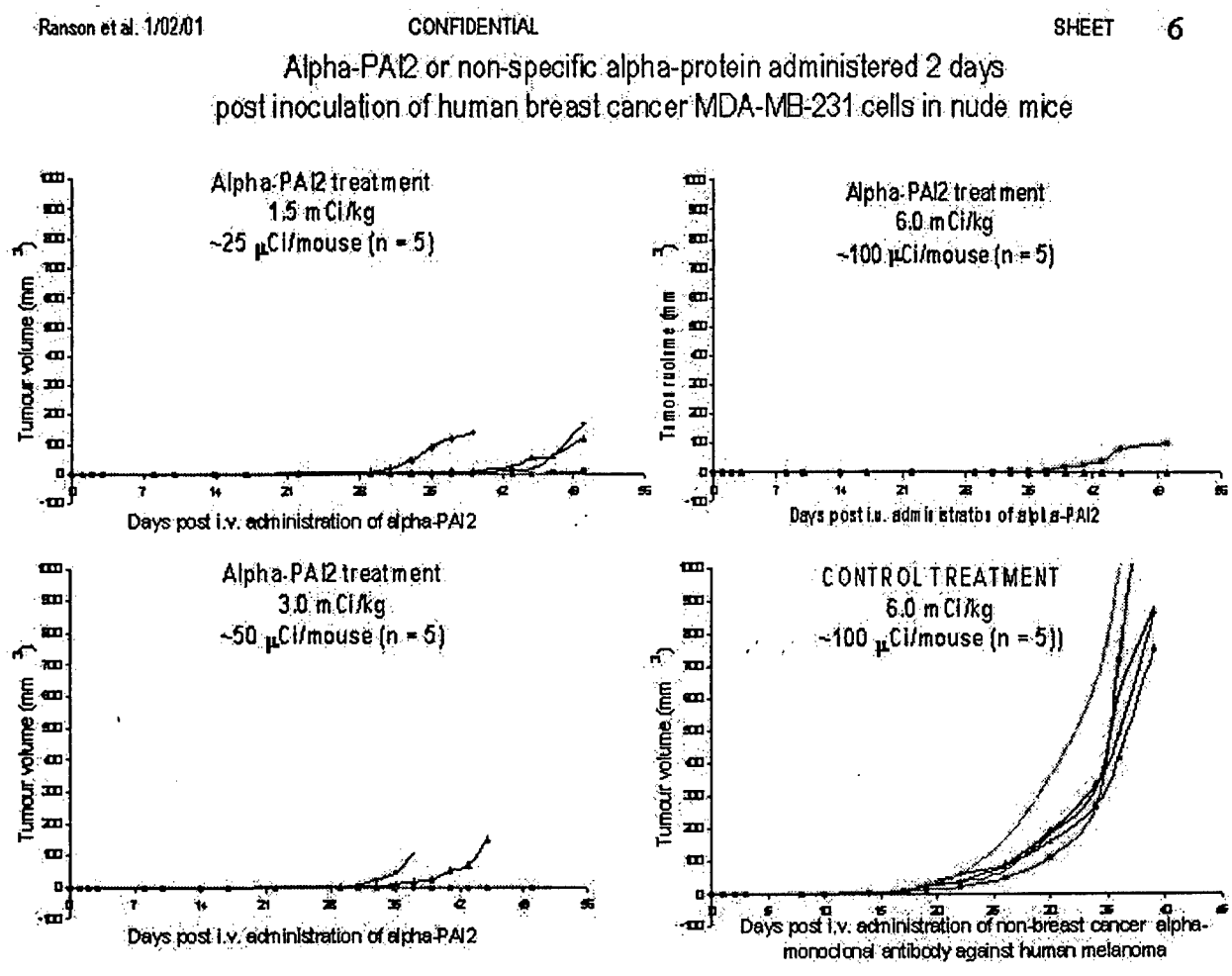

Figure 4. Effect on tumour growth after systemic (ip) administration of alpha-PAI2 at 2 days post-inoculation of breast cancer cells. An indicative dose response is observed, with 4/5 tumours being controlled at 6 mCi/kg.

KEY RESEARCH ACCOMPLISHMENTS

1 In vivo toxicity measurements show that therapeutic doses of alpha PAI2 up to 6 mCi/kg bodyweight can be delivered to mice without significant weight loss.
2 Complete inhibition of the growth of breast cancer tumours has been achieved by local alpha-PAI2 injection at 2 days post-inoculation.
3 Inhibition of tumour growth established for single systemic (ip) TAT.
4 In vitro results accepted for publication.
5 Registration of international patent.

REPORTABLE OUTCOMES

Manuscripts in press:

Allen et al: "In vitro and preclinical targeted alpha therapy for melanoma, breast, prostate and colorectal cancers". Reviews in Hematology and Oncology.

Ranson et al, "In vitro cytotoxicity of Bismuth-213 labelled plasminogen activator inhibitor type 2 on human breast cancer cells. Breast cancer Research and Treatment, in press

Manuscript in preparation:

B J Allen, S Rizvi, Y Li, M Ranson: "In vitro and preclinical studies of targeted alpha therapy for human breast cancer using ^{213}Bi-labeled plasminogen activator type 2".

Patent applied for and/or issued:

Australian provisional patent number PQ5824, file date 24/2/2000, expiry date 24/2/2001 "A method of treatment and agents therein for cancer". Filed in the name of Biotech Australia Pty Ltd, University of Wollongong, Medical Scitec Australia Pty Ltd (attached). Full international patent is being registered.

Funding applied for based on work supported by this award:

USDOD CTR Grant Application for 2002-4: "Development and implementation of alpha-PAI2 for targeted alpha therapy of breast cancer."

Principle Investigators: B J Allen (St George Hospital & University of Wollongong), M Ranson (University of Wollongong), M Links (St George Hospital), C L Bunn (Biotech Australia).

Annual Report Assistance Award No. DAMD17-99-1-9383

This was a successful pre-proposal BC014016, which is now in the full submission stage.

USDOD Grant Application for 2002-4: "Preclinical trials of multi-targeted alpha therapy for the control of micrometastatic prostate cancer". B J Allen (St George Hospital), P J Russell (Prince of Wales Hospital), M Ranson (University of Wollongong), P Cozzi (St George Hospital)

CONCLUSIONS

The research so far provides an even stronger case for the use of a new targeting protein, PAI2, to target breast cancers cells and the alpha emitter Bi-213 to kill these targeted cells. As such, we are clearly moving towards the development of a new therapeutic modality for breast cancer.

If ultimately successful, alpha-PAI2 therapy can change the prognosis for many breast cancer patients who are clinically free of disease but who have a high probability of micrometastatic disease that would eventually lead to a reduced life span. We are now well advanced in our preclinical studies, the next step being the investigation of sequential dose treatment model as a prelude to clinical application.

REFERENCES

1. Allen BJ. Can alpha immunotherapy succeed where other systemic therapies have failed? Nucl Med Commun. 20: 205-207, 1999.
2. American Cancer Society (ACS): News Today of Breast Cancer Resource Center, June 28, 2000.
3. Raso V. The magic bullet-nearing the century mark. Semin. Cancer Biol. 1: 227-243, 1990.
4. Allen BJ. Targeted alpha therapy: evidence for efficacy of alpha-immunoconjugates in the management of micrometastatic cancer. Australas Radiol. 43: 480-486, 1999.
5. Allen BJ and Blagojevic N. Alpha and beta emitting radiolanthanides in targeted cancer therapy. Nucl Med Commun. 17: 40-47, 1996.
6. Humm JL. A microdosimetric model of astatine-211 labeled antibodies for radioimmunotherapy. Int J Radiat Oncol Biol Phys. 13: 1767-1773, 1987.
7. Bloomer WD, McLaughlin WH, Lambrecht RM, Mirzadeh S, Madara JL, Milius RA, Zalutsky MR, Adelstein SJ, Wolf AP. ^{211}At radiocolloid therapy: further observations and comparision with radiocolloids of ^{32}P, ^{165}Dy and ^{90}Y. Int J Radiat Oncol Biol Phys.10: 341, 1984.
8. Macklis RM, Beresford BA, Palayoor S, Sweeney S, Humm JL. Cell cycle alterations, apoptosis, and response to low-dose-rate radioimmunotherapy in lymphoma cells. Int J Oncol. 2: 711-715, 1993.
9. Horak E, Hartmann F, Garmestani K, Wu C, Brechbiel M, Gansow OA, Landolfi NF, Waldmann TA. Radioimmunotherapy targeting of HER2/neu oncoprotein on ovarian tumour using lead-212-DOTA-AE1. Nucl. Med. 38: 1944-1950, 1997.
10. Larsen RH, Akabani G, Welsh P, Zalutski MR. The cytotoxicity and microdosimetry of astatine-211-labeled chimeric monoclonal antibodies in human glioma and melanoma cells in vitro. Radiation Res. 149: 152-157, 1998.
11. McDevitt MR, Sgouros G, Finn RD, Humm JL, Jurcic JG, Larson SM, Scheinberg DA. Radioimmunotherapy with alpha-emitting nuclides. Eur J Nucl Med. 25: 1341-1351, 1998.
12. McDevitt MR, Finn RD, Sgouros G, Ma D, Scheinberg DA. An ^{225}Ac / ^{213}Bi generator system for therapeutic clinical applications: construction and operation. Appl Rad and Isot. 50: 895-904, 1998.
13. Van Geel JNC, Fuger J, Koch L. Verfahern zur erzeugung von actinium-225 und Bismuth-213 European Patent No. 0 443 479 B1. 1994.
14. Boll RA, Mirzadeh S, Kennel SJ, DePaoli DW, Webb OF. ^{213}Bi for alpha-particle-mediated radioimmunotherapy. J. Label. Compds. Radiopharm. 40:341, 1998.
15. Nikula TK, McDevitt MR, Finn RD et al. Alpha-emitting bismuth cyclohexylbenzyl DTPA constructs of recombinant humanized anti-CD33 antibodies: pharmacokinetics, bioactivity, toxicity and chemistry. J. Nucl. Med. 40:166-176, 1999.
16. Kennel SJ, Stabin M, Roeske JC, Foote LJ, Lankford PK, Terzaghi-Howe M, Patterson H, Barkenbus J, Popp DM, Boll R, Mirzadeh S. Radiotoxicity of bismuth-213 bound to membranes of monolayer and spheroid cultures of tumor cells. Radiat Res 151: 244-256,1999
17. Kennel SJ, Boll R, Stabin M, Schuller HM and Mirzadeh S. Radioimmunotherapy of micrometastases in lung with vascular targeted ^{213}Bi. Br J Cancer 80: 175-184, 1999.
18. Rizvi SM, Sarkar S, Goozee G, Allen BJ. Radioimmunoconjugates for Targeted Alpha Therapy of Malignant Melanoma. Melanoma Res. 10: 281-290, 2000.
19. Adams GP, Shaller CC, Chappell LL, Wu C, Horak M, Simmons HH, Litwin S, Marks JD, Weiner LMN, Brechbiel MW. Delivery of the alpha-emitting radioisotope bismuth-213 to solid tumours by a single-chain Fv and diabody molecules. Nucl Med Biol. 27: 339-346, 2000
20. McDevitt MR, Barendswaard E, Ma D et al. An alpha-particle emitting antibody (^{213}Bi-J591) for radio-immunotherapy of prostate cancer. Cancer Res. 60:6095-6100, 2000

21. Rizvi SMR, Allen BJ, Tian Z, Sarkar S. In vitro and preclinical studies of targeted alpha therapy for colorectal cancer. Colorectal Disease, in press.
22. Rizvi SMR, Henniker AJ, Goozee G, Allen BJ. In vitro testing of the leukaemia monoclonal antibody WM-53 labeled with alpha and beta emitting radioisotopes. Leukaemia Research, in press.
23. Pollanen J, Stephens R, Vaheri A. Directed plasminogen activation at the surface of normal and malignant cells. Adv. Cancer Res. 57: 273-328, 1991.
24. Andreasen PA, Kjoller L, Christensen L, and Duffy M. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 72: 1-22, 1997.
25. Schmitt M, Wilhelm OG, Reuning U, et al. The urokinase plasminogen activation system as a novel target for tumour therapy. Fibrinol. & Proteol. 14: 114-132, 2000.
26. Schmitt M, Janicke F, Chucholowski N, and Pache L and Graeff H. Tumour-associated urokinase-type plasminogen activator: biological and clinical significance. Biol. Chem. Hoppe-Seyler. 373:611-622, 1992.
27. Yamamoto M, Sawaya R, Mohanam S et al. Activities, localizations, and roles of serine proteases and their inhibitors in human brain tumour progression. J Neuro-Oncology 22:139-151, 1994
28. Mustojoki S, Alitalo R, Stephens RW, and Vaheri A. Plasminogen activation in human leukemia and in normal hematopoietic cells. APMIS. 107:144-149, 1999.
29. Dublin E, Hanby A, Patel NK, Liebman R, and Barnes D. Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma – fibroblastic expression has strong associations with tumour pathology. American J. Pathol. 157:1219-1227, 2000.
30. Bianchi E, Cohen RL, Thor AT et al. The urokinase receptor is expressed in invasive breast cancer but not in normal breast tissue. Cancer Res. 54:861-866, 1994.
31. Del Vecchio S, Stoppelli MP, Carriero MV et al. Human urokinase receptor concnetratin in malignant and benign breast tumours by in vitro quantitative autoradiography: comparison with urokinase levels. Cancer Res. 53:3198-3206, 1993.
32. Christensen L, Wiborg Simonsen AC, Heegaard CW, et al. Immunohistochemical localization of urokinase-type plasminogen activator, type-1 plasminogen-activator inhibitor, urokinase receptor and a2-macroglobulin receptor in human breast carcinomas. Int. J. Cancer. 66:441-452, 1996.
33. Constantini V, Sidoni A, Deveglia R et al. Combined overexpression of urokinase, urokinase receptor, and plasminogen activator inhibitor-1 is associated with breast cancer progression. Cancer 77:1079-1088, 1996
34. Fisher JL, Field CL, Zhou H et al. Urokinase plasminogen activator system gene expression is increased in human breast carcinoma and its bone metastases – a compariosn of normal breast tissue, non-invasive and invasive carcinoma and osseous metastases. Br. Cancer Res. Treat. 61:1-12, 2000.
35. Kruithof EKO, Baker MS, Bunn CL. Biochemistry, cellular and molecular biology and clinical aspects of plasminogen activator inhibitor type-2. Blood 86: 4007-4024, 1995.
36. Hang MTN, Ranson M, Saunders DN, Liang XM, Bunn CL, Baker MS. Pharmacokinetics and biodistribution of recombinant human plasminogen activator inhibitor type 2 (PAI-2) in control and tumour xenograft-bearing mice. Fibrinol. and Proteol. 12: 145-154, 1998.
37. Andronicos NM, Ranson M, Bognacki J, Baker MS. The human ENO1 gene product (recombinant human alpha-enolase) displays characteristics required for a plasminogen binding protein. Biochimica et Biophysica Acta. 1337: 27-39, 1997.
38. Boll RA, Mirzadeh S and Kennel SJ. Optimizations of radiolabeling of immunoproteins with ^{213}Bi. Radiochimica Acta 79: 145-149, 1997.
39. Paik CH, Ebbert MA, Murphy PR, Lassman CR, Reba RC, Eckelman WC, Pak KY, Powe J, Steplewski, Koprowski. Factors influencing DTPA conjugation with antibodies by cyclic DTPA anhydride. J Nucl Med. 24: 1158-1163, 1983.

40. Bennett KL and Sheil MM. Probing the coordination of metal ions by diethylenetriaminepentacetic acid-conjugated proteins with electrospray ionisation mass spectrometry. Eur. Mass Spectrum 3:233-244, 1997.
41. Ranson M, Andronicos N, O'Mullane and Baker MS. Increased plasminogen binding is associated with metastatic breast cancer cells: differential expression of plasminogen binding proteins. Br J Cancer 77: 1586-1597, 1998.
42. Laug WE, Cao XR, Yu YB, Shimada H, Kruithof EKO. Inhibition of invasion of HT1080 sarcoma cells expressing recombinant plasminogen activator inhibitor 2. Cancer Res. 53: 6051-6057, 1993.
43. Mueller BA, Yu YB, Laug WE. Overexpression of plasminogen activator inhibitor 2 in human melanoma cells inhibits spontaneous metastasis in scid/scid mice. Proc Natl Acad Sci USA 92: 205-209, 1995.
44. Evans DM, Lin PL. Suppression of pulmonary metastases of rat mammary cancer by recombinant urokinase plasminogen activator inhibitor. American Surgeon 61: 692-696, 1995.
45. Yang J-L, Seetoo D Wang Y, Ranson M, Berney CR, Ham JM, Russell PJ, Crowe PJ. Urokinase-type plasminogen activator and its receptor in colorectal cancer: independent prognostic factors of metastasis and cancer specific survival, and potential therapeutic targets. Intl J. Cancer 89:431-439, 2000.
46. Jankun J. Antitumour activity of the type 1 plasminogen activator inhibitor and cytotoxic conjugate in vitro. Cancer Res. 52: 5829-5832, 1992.
47. Jankun J. Targeting of drugs to tumours: The use of the plasminogen activator inhibitor as a ligand. Targeting of Drugs 4. Edited by Gregoriadis G et al. Plenum Press, New York, pp. 67-79, 1994.
48. Y Li, Z Tian, N H Bander, B J Allen, In vitro and in vivo studies of targeted alpha therapy of human prostate cancer with Bi-213 labeled J591 antibody against the prostate specific membrane antigen. Prostate Cancer and Prostatic Disease, submitted.
49. M Ranson, Z Tian, B J Allen, N M Andronicos, S Rizvi, In vitro cytotoxicity study of human breast cancer cells using Bi-213 labeled plasminogen activator type 2. Breast Cancer Research and Treatment, submitted

APPENDICES

1 Abstract: In vitro and preclinical studies of targeted alpha therapy for human breast cancer using ^{213}bi-labeled-plasminogen activator inhibitor type 2

2 Paper by Ranson et al, Breast Cancer Research and Treatment, in press.

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IN VITRO AND PRECLINICAL STUDIES OF TARGETED ALPHA THERAPY FOR HUMAN BREAST CANCER USING ^{213}BI-LABELED-PLASMINOGEN ACTIVATOR INHIBITOR TYPE 2

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Abstract

Many breast cancer patients receive minimal long-term survival benefit despite undergoing surgery and adjuvant therapy, indicating that micrometastases are not eradicated. We are developing a new adjuvant therapy for the control of metastatic breast cancer based on alpha-emitting nuclides. This therapy exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells. Once bound to specific cell-surface receptors, uPA efficiently activates plasminogen to the broad-spectrum protease, plasmin. A large body of experimental and clinical evidence implicates the uPA system as a marker of malignancy and therefore a useful and accessible specific target for therapy.

We have successfully labeled and tested recombinant human PAI2 with the alpha radioisotope ^{213}Bi to produce alpha-PAI2. Low doses of alpha-PAI2 are highly cytotoxic towards breast cancer cell lines in vitro, whereas non-specific alpha-BSA had no cytotoxic effect, reflecting that non-targeted cells are immune from alpha-PAI2.

In vivo toxicity studies in nude mice show that up to 6 mCi/kg of alpha-PAI2 (ip) is well tolerated. In vivo efficacy experiments in nude mice demonstrate in 5/5 mice that a local injection of alpha-PAI2 can completely inhibit the growth of tumour at 2 days post-cell inoculation. Further, systemic (iv) administration of alpha-PAI2 at 2 days post-inoculation can also cause tumour growth inhibition in a dose dependent manner, with 3/5 tumours uncontrolled at 1.5 mCi/kg, 2/5 at 3 mCi/kg and 1/5 at 6 mCi/kg. Thus alpha-PAI2 is successful in targeting isolated cells and preangiogenic cell clusters.

These results indicate the promising potential of alpha-PAI2 as a novel therapeutic agent for micrometastatic breast cancer.

Key words:

Bismuth-213 (^{213}Bi), breast cancer cells, plasminogen activator inhibitor type 2 (PAI2), urokinase, targeted alpha therapy.

In vitro cytotoxicity study of human breast cancer cells using Bismuth-213 (^{213}Bi)-labeled-plasminogen activator inhibitor type 2 (alpha-PAI-2)

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ABSTRACT

Many breast cancer patients receive minimal long-term survival benefit despite undergoing surgery and adjuvant therapy, indicating that micrometastases are not eradicated. We are developing a new adjuvant therapy for the control of metastatic breast cancer based on alpha-emitting nuclides. This exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells. Once bound to specific cell-surface receptors, uPA efficiently activates plasminogen to the broad-spectrum protease, plasmin. A large body of experimental and clinical evidence implicates the uPA system as a marker of malignancy and therefore a useful and accessible specific target for therapy. Surface receptor-bound uPA can be efficiently inhibited by its natural inhibitor, plasminogen activator inhibitor type 2 (PAI-2). We have successfully labeled recombinant human PAI-2 with the alpha radioisotope ^{213}Bi to produce alpha-PAI-2. Labeling efficiencies of up to 95% are obtained, the alpha-PAI-2 is stable in serum and the uPA inhibitory activity is maintained as determined by complex formation with uPA and by inhibition of uPA activity. Low doses of alpha-PAI-2 are highly cytotoxic towards breast cancer cell lines in vitro. This effect is specific as uPA-blocking agents that effectively inhibit PAI-2 binding significantly improves cell survival by a factor of 2.5, while non-specific alpha-BSA had no cytotoxic effect. Furthermore, no cytotoxicity of alpha-PAI-2 was observed with freshly isolated normal human leukocytes reflecting that non-targeted cells are immune from alpha-PAI-2. In conclusion, we have validated, in vitro, the potential of alpha-PAI-2 as a novel therapeutic agent for breast cancer.

Key words:

Bismuth-213 (^{213}Bi) Breast cancer cells Plasminogen activator inhibitor type 2 (PAI-2) Urokinase Targeted alpha therapy

INTRODUCTION

The major failure in breast cancer management is due to the incomplete killing of invisible malignant tumour cells that have spread throughout the body [1]. Despite the many treatments available, such as surgery, radiation therapy, hormone therapy and chemotherapy, the control of metastases of breast cancer remains elusive. The American Cancer Society estimates 182,800 new cases of invasive breast cancer in the year 2000 among women in America, and 40,800 are expected to die from the disease [2]. Novel, more effective treatments that overcome this problem in breast cancer management are essential. Targeted therapy, first discussed over 100 years ago by Paul Erhilch, is based on the idea that a drug will attack its target without damaging other tissue [3]. Targeted alpha therapy (TAT) uses an alpha emitting radionuclide as a lethal medicament via an effective targeting carrier to kill cancer cells [4]. We are investigating a novel TAT approach that exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells.

Alpha emitting radionuclides emit alpha particles with energies up to an order of magnitude greater than most of beta rays, yet their ranges are two orders of magnitude less as alpha particles have a linear energy transfer (LET) which is about 100 times greater [5]. This is manifested by a high relative biological effectiveness (RBE). As a result, a much greater fraction of the total energy is deposited in cells with alphas and very few nuclear hits are required to kill a cell. Consequently, 100-fold enhancement in radiation dose [6, 7] would be delivered to the nucleus of a breast cancer cell if an effective carrier is employed to take the alpha radionuclide into that cancer cell. So only alphas have the possibility to kill the metastatic cancer cells at tolerable dose limits, whereas the low LET of betas make this a very difficult task within human dose tolerance limits.

Availability of the alpha emitting radionuclides has been the major problem in the past for their large-scale scientific and clinical application. Studies have been carried out on ^{211}At and ^{212}Bi [8, 9, 10, 11] with encouraging results. The therapeutic potential of the alpha nuclide, ^{213}Bi, has been

studied in the treatment of single cell neoplastic disorders such as leukemia, lymphoma and micrometastatic neoplasm [4, 12]. The stable and reliable ^{225}Ac generator of ^{213}Bi has been produced, modified and used in laboratories successfully [13, 14, 15, 16, 17,18,19]. Our group has modified methods of conjugating ^{213}Bi radionuclide to antibodies with the stable chelator cyclic anhydride of diethylenetriaminepentacetic acid (cDTPA) for use in the alpha therapy of melanoma [16].

A large body of experimental and clinical evidence implicates the uPA system not only as a modulator of the aggressive behaviour of tumour cells but also as a strong prognostic factor for predicting poor breast cancer patient outcome [20]. Under physiological conditions, most cells express very little or no uPA and/or its specific cell surface receptor (uPAR) [21]. Once bound to uPAR, uPA efficiently activates plasminogen to the broad spectrum, extracellular matrix-degrading protease, plasmin [20,21]. Plasmin also activates zymogen metalloproteases which more thoroughly degrades the collagen structural components and releases growth factors which induce the synthesis of proteolytic factors [21,22].

The activity of uPA is physiologically regulated by plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2) [21,22,23]. PAI-2, a member of the serpin (serine proteinase inhibitor) family, is produced by many cell types as either a predominantly intracellular non-glycosylated form (43-47 kDa) or as an extracellular glycosylated form (60kDa). Unlike many other serpins both PAI-2 forms are insensitive to oxidative inactivation [23]. PAI-2 is an efficient inhibitor of active uPA forming SDS-stable 1:1 complexes with uPA (rate constant 10^6 $M^{-1}S^{-1}$) [23]. Cell surface-bound uPA is accessible to and inhibitable by PAI-2 [23, 24], and a number of studies have suggested the potential for PAI-2 to inhibit cancer cell invasion and metastasis [23]. The pharmacokinetics and biodistribution of human recombinant ^{125}I-labelled PAI-2 in both control and animals bearing human colon cancer (uPA-positive HCT116 cell line) xenografts has been established [24]. Briefly,

^{125}I-PAI-2 localised in 0.5 cm^3 tumour xenografts quickly (after 1 min, peaking at 30 - 60 min at approx. 1.5% of total injected dose). While the majority of radioactivity localised to the major organs after 5 min, radioactivity was cleared more quickly from these organs compared to tumours. Furthermore, repeat intravenous, sub-cutaneous, or intra-peritoneal injections of ^{125}I-PAI-2 resulted in an accumulation of radioactivity without an accompanying increase in the major organs or in toxicity. In addition, tumour associated ^{125}I-PAI-2 correlated with tumour mass. Since ^{125}I -PAI-2 specifically bound to HCT116-associated uPA [23, 24], it is likely that exogenously administered PAI-2 targets uPA expressing tumours.

It is clear that uPA is a specific malignant breast cancer cell marker and that PAI-2 represents a useful targeting agent. In this paper we report for the first time, the production and characterization of a new alpha-nuclide emitting cytotoxic agent ^{213}Bi-labeled PAI-2 (alpha-PAI-2). The specificity of alpha-PAI-2 cytotoxicity for two human breast cancer cell lines in vitro indicates that alpha-PAI-2 is a very promising new therapeutic modality for the control of tumour metastases by killing targeted cancer cells.

MATERIALS AND METHODS

Materials

Glu-plasminogen was purified from human plasma as described by Andronicos et al [25]. Human recombinant PAI-2 (47 kDa) was provided by Biotech Australia Pty Ltd. Microspin concentrators were purchased from Millipore (Bedford, MA, USA). RPMI-1640 was purchased from Life Technologies (Castle Hill, NSW, Australia). Fetal calf serum (FCS) was obtained from Trace Bioscientific (Castle Hill, NSW, Australia). The cyclic anhydride of diethylenetriaminepentacetic acid (cDTPA) was purchased from Aldrich Chemical Company. Bovine serum albumin (fraction V) (BSA) and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO. USA). Human urokinase plasminogen activator (uPA) was purchased from Serono (Sydney, NSW, Australia). Glu-gly-arg chloromethylketone (EGR-CMK) was purchased from Calbiochem (Sydney, NSW, Australia). Spectrozyme-UK, mouse anti-human uPA IgG_1 (#394), mouse anti-human uPAR IgG_{2a} (#3696), and mouse anti-human PAI-2 IgG_1 (#3750) monoclonal antibodies were purchased from American Diagnostica Inc (Greenwich, CT, USA). Mouse isotype control subclasses IgG_1, IgG_{2a} antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG were from Silenus (Sydney, NSW, Australia). Fresh human leukocytes were isolated from whole blood using the Fycoll-Plaque (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions.

Radioisotope

Alpha particle emitting radionuclide, ^{213}Bi, was produced from the $^{225}Ac/^{213}Bi$ generator which was purchased from the United States Department of Energy. ^{213}Bi was eluted from the ^{225}Ac column with 250 µL of freshly prepared 0.15 M distilled and stabilized hydriodic acid followed by washing with 250 µL sterile distilled water [16]. The first elution was not used, and a time of 2-3 h was allowed for ^{213}Bi to regenerate on the column for the next elution. For all activity calculations corrections were made for ^{213}Bi decay using the half-life of 45 min.

PAI-2 conjugation with cDTPA

PAI-2 and BSA were conjugated with cDTPA by a modification of the method described by Rizvi et al. [16], according to Paik et al. and Boll et al. [26, 27]. PAI-2/BSA (1 mg) dissolved in PBS was conjugated to cDTPA by first increasing the pH to approximately 8.2 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). A 50 fold molar excess of cDTPA was added and the reaction mixture incubated at 25°C for 1 h with intermittent rocking. Three reaction volumes of PBS were used to purify DTPA-labeled proteins away from free cDTPA using a microspin concentrator.

The concentrations of DTPA-labeled PAI-2/BSA were measured by BIORAD DC protein assay reagent kit. The binding activity of DTPA-labeled PAI-2 was examined by its ability to form complexes after 30 min incubation with equimolar amounts of active human uPA at room temperature [24]. The uPA/conjugated PAI-2 complexes were detected by SDS-PAGE (12% non-reducing gel). The uPA inhibitory activities of DTPA-PAI-2 and FITC-PAI-2 were measured using the colour substrate for uPA, carobenzoxy-L-γ-glutamyl-(α-t-butoxy)-glycyl-arginine-p-nitroanilide-diacetate Spectrozyme-UK (conditions described in the figure legend 1 and 2). Standard curves were constructed using uPA.

^{213}Bi labeling of cDTPA-PAI-2

Concentrated DTPA-PAI-2 stocks were diluted with 500 mM sodium acetate at pH 5.5 and labeled with free ^{213}Bi for 20 min at room temperature. After labeling, ^{213}Bi-cDTPA-PAI-2 (alpha-PAI-2) was buffer exchanged into PBS using a PD-10 column using PBS (pH 7.0) as the eluting buffer. ^{213}Bi-cDTPA-BSA (alpha-BSA) was radiolabeled by similar methods. The radiolabeling efficiency was determined by Instant Thin Layer Chromatography (ITLC) using a 10 μL aliquot of the final reaction mixture applied to Gelman paper (strip size 1 x 9cm, Gelman Science, Ann Arbor, MI). The paper strips were developed using 0.5 M sodium acetate (pH 5.5) as the solvent. The paper strips were cut into four sections and the gamma emissions from the radioisotope along the paper

were counted using a 340-540 keV window. The radiolabeled protein is found at the origin section, while free radioisotope is found at the solvent front section.

The stability of ^{213}Bi labeled protein conjugates in human serum was determined by incubation in fresh human serum at 37 °C. At 0 and 45 min, 10 μL samples were withdrawn and subjected to ITLC. The origin activity reflects the stability of radiolabeled protein conjugates. The solvent front activity reflects the leaching of radiolabeled protein conjugates.

Cell culture

The metastatic MDA-MB-231 and non-metastatic MCF-7 human breast cancer cell lines [28] were routinely cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS and passaged using Trypsin/EDTA. The cells were incubated in a humidified incubator at 37 °C with a 5% carbon dioxide air atmosphere.

For all experimental procedures, sub-confluent cells that had been in culture for 48 h without a change of media were harvested by rinsing flasks twice with PBS (pH 7.2) and then detaching with PBS/0.5mM EDTA at 37 °C for 5 min. Cells were collected and resuspended in the appropriate buffer as described below.

Flow cytometry

For the detection of cell-surface uPA, uPAR and PAI-2 indirect immunofluorescence staining was performed as described by Ranson et al. [28]. Briefly, cells were incubated with either an irrelevant isotype control or anti-human uPA, uPAR and PAI-2 monoclonal antibody for 30 min on ice (10 μg mL^{-1} in cold RPMI/0.1% BSA). The cells were washed with 1 mL of cold RPMI/0.1% BSA and incubated with FITC-conjugated anti-mouse IgG (1:50 dilution of stock in cold RPMI/0.1% BSA) for 30 min on ice in dark. The cells were washed again and resuspended in 0.5 mL cold PBS/0.1% sodium azide containing 5 μg mL^{-1} propidium iodide (PI). Cell-associated fluorescence was then measured by dual-colour flow cytometry. This technique was used to

establish 'gates'- the exclusion of PI for a viable 'gate', the inclusion of PI for a non-viable 'gate' [28].

All flow cytometry data were analysed using CELLQuest software (Becton-Dickinson). Autofluorescence or isotype control fluorescences were subtracted from all flow cytometry experiments. Only cell-surface associated (viable) cell fluorescence is reported.

Immunohistochemistry

Cells grown on glass coverslips for 48 h in growth medium, were washed three times with PBS at room temperature, fixed by incubation with PBS supplemented with 1% gluteraldehyde for 1 h at room temperature, and then washed once with PBS. Endogenous peroxidases were inactivated with 3% H_2O_2 for 5 min at room temperature. The cells were incubated with 10% human serum for 30 min, washed with PBS, and then incubated for 1 h with primary antibodies diluted with PBS/10 % human serum (final concentration 20 μg/mL) at room temperature. The cells were washed twice with PBS and incubated with the secondary reagents from the DAKO LSAB+ Kit according to the manufacturer's instructions. After rinsing with PBS, the cells were viewed using a video camera (National Panasonic) attached to an inverted compound microscope (Leica, Germany). Images of the cells (original magnification x400 unless otherwise stated) were captured by a Power PC (Macintosh 8500/20) using Apple Video Player software (Macintosh).

Cytotoxicity assay

The CellTiter 96 Aqueous non-radioactive cell proliferation assay (Promega, WI, USA) was used to determine the effect of ^{213}Bi labeled proteins on cell survival. The CellTiter 96 Aqueous solution reagent is composed of the tetrazolium compound (MTS) and an electron-coupling reagent, phenazine ethosulfate (PES).

The activities of alpha-PAI-2 and alpha-BSA preparations were measured using a radioisotope calibrator and neutralized to pH 7.0 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). Immediately after this, five serial doses of alpha-PAI-2 and one dose of alpha-BSA were

prepared in 100 µL RPMI/10%FCS and added to 96-well plates in triplicate containing 20,000 cells/well in 100 µL RPMI medium/10% FCS. The plates were then incubated overnight in a 5% carbon dioxide atmosphere at 37 °C. Controls were performed in triplicate in the same 96-well plate for each experiment and consisted of RPMI/10% FCS medium alone. In some cases further controls were performed as described in the legend to Table 1. In all cases the cells had been previously pre-incubated with 20 µg/mL plasminogen for 20 min at room temperature and washed before placing into 96-well plates. In some cases, the cells were also treated with 0.5 mM EGR-CMK (a specific uPA inhibitor) [24] for 15 min at room temperature after plasminogen activation and prior to incubation with radiolabeled proteins.

The cells were then washed and incubated with 100 µL phenol-red free RPMI without FCS containing 20 µL of the CellTiter 96 Aqueous One Solution reagent. After 3 h incubation in a 5% carbon dioxide atmosphere at 37 °C, the reaction was stopped by the addition of 10% SDS, and the absorbance in each well was recorded at 490 nm using a SPECTROmax plate reader (BIORAD). The absorbance reflects the number of surviving cells. Blanks were subtracted from all data and analysed using Prism software (GraphPad Software Inc, USA).

RESULTS

cDTPA-PAI-2 retains its uPA inhibitory activity

The incubation of uPA with PAI-2 in a 1:1 molar ratio forms a SDS-stable uPA-PAI-2 complex that is indicative of the inhibitory activity of the molecule [24]. Thus, SDS-PAGE can be used to determine if the uPA inhibitory activity of cDTPA-PAI-2 was retained after modification. As demonstrated in Figure 1 both unlabeled PAI-2 and cDTPA conjugated PAI-2 were able to form SDS-stable complexes with both high molecular weight uPA (98 kDa) and active low molecular weight uPA (80 kDa). Quantitatively, the uPA inhibitory capacity was indirectly determined using the SPECTROZYME-UK assay. No significant difference was observed between the uPA inhibitory activity of cDTPA-PAI-2 and unmodified PAI-2 (Figure 2). Thus, the conjugation of PAI-2 with cDTPA did not significantly perturb the ability of PAI-2 to inhibit the activity of uPA.

^{213}Bi radiolabeling of cDTPA-PAI-2 (alpha-PAI-2)

The radiolabeling reaction efficiency of alpha-PAI-2 was determined by ITLC (Figure 3A). Greater than 90% of the radioactivity due to alpha-PAI-2 stays at the origin (the 1st fraction). In contrast, greater than 90% of the free isotope ^{213}Bi migrates to the solvent front of the ITLC strip (the 4th fraction). The specific activity of the alpha-PAI-2 was approximately 10-15 μCi/μg.

When incubated in human serum for 45 min (half-life of ^{213}Bi), less than 10% of the label leached from the alpha-PAI-2 sample as determined by the amount of radioactivity in the 4th fraction compared to the total radioactivity (Figure 3B).

Cell surface expression of endogenous uPA, uPAR and PAI-2 on human breast cancer cell lines

The characteristic high and low cell surface levels of endogenous uPA and uPAR expression on MDA-MB-231 and MCF-7 cells, respectively [28], were confirmed by dual colour flow cytometry (Figure 4 A) and immunohistochemistry (Figure 4B). The endogenous cell surface levels of PAI-2 were also examined on these cell lines by flow cytometry (Figure 4A). While the uPA and

uPAR antigen levels were approximately 28-fold and 6-fold higher on the metastatic MDA-MB-231 cells compared to the non-metastatic MCF-7 cells, the endogenous levels of cell surface associated PAI-2 were comparably low on both cell lines (Figure 4A). Despite the low cell surface PAI-2 levels, both cell lines produce intracellular PAI-2 as detected by western blotting of whole cell lysates (data not shown).

Immunohistochemistry mirrored the endogenous uPA and uPAR antigen expression differences seen in the two cell lines by flow cytometry (Figure 4B). Notably, the staining patterns of both antigens for both cell lines were punctuate and heterogeneous. Most of the MDA-MB-231 cells were highly positive for either antigen with less than 10% being weakly positive. In contrast, most of the MCF-7 cells were only weakly positive with less than 10% being moderately positive.

Alpha-PAI-2 is highly cytotoxic towards the MDA-MB-231 and MCF-7 cell lines

Cell survival experiments of MDA-MB-231 and MCF-7 cell lines showed high cytotoxicity with alpha-PAI-2 as shown in Figure 5 but negligible effect for controls (alpha-BSA, cDTPA-PAI-2 or PAI-2 only) in Table 1. The D_o (37% cell survival) values with alpha-PAI-2 were calculated to be 2.1 ± 0.2 μCi and 2.3 ± 0.2 μCi for MDA-MB-231 and MCF-7 cell lines, respectively. These results were averaged from 6 experiments in triplicate. Notably, the D_o values of cells pre-treated with uPA specific inhibitor EGR-CMK prior to incubation with alpha-PAI-2 increased to 5.3 ± 0.2 μCi (n=2) and 5.1 ± 0.2 μCi (n=2) for MDA-MB-231 and MCF-7, respectively. At the maximum dose of alpha-PAI-2 (5 μCi) cell survival was reduced to 11-13% for both cell lines. In comparison, at this maximum dose of alpha-PAI-2 the survival of EGR-CMK pre-treated cells increased to approximately 40% (Table 1). The cytotoxicity of alpha-BSA, PAI-2 or cDTPA-PAI-2 was also examined (Table 1). Alpha-BSA (5 μCi) in the presence or absence of EGR-CMK had a minimal effect on survival of the MDA-MB-231 and MCF-7 cells. MDA-MB-231 and MCF-7 cell survival was unaffected by incubation with PAI-2 or cDTPA-PAI-2 (Table 1). In addition, the cell survival

percentages of normal human leukocytes treated with alpha-PAI-2 with or without EGR-CMK were all more than 90% (Table 1).

DISCUSSION

A number of novel techniques that target the uPA system for tumour therapy have been suggested and are being investigated [20]. While a large body of clinical data indicates that high levels of uPA, uPAR and PAI-1 can be used to predict poor patient prognosis for multiple types of solid tumors [20], the significance of PAI-2 in predicting outcome of cancer patients is unclear. Some studies have shown that a high PAI-2 antigen level in carcinoma is prognostic for a poor overall survival of patients with either colorectal cancer or breast cancer [29,30,31]. Other studies have shown that elevated PAI-2 in primary breast tumors is indicative of a good prognosis [32]. Still other studies have found no correlation between patient survival and PAI-2 levels in colorectal cancer patients [33]. In this study, the low level of cell surface PAI-2 compared to higher levels of uPA/uPAR on the breast cancer cell lines indicates that most of cell-associated uPA would be accessible to exogenous PAI-2.

The ability of PAI-2 to target cancer cells has been demonstrated by several laboratories. For example, extracellular matrix degradation by colon cancer cells *in vitro* is inhibited by recombinant PAI-2 [34], local invasion of human sarcoma xenografts is limited by stable expression of PAI-2 in the sarcoma cells [35], over-expression of PAI-2 in human melanoma cells inhibits spontaneous metastasis in immunocompromised mice [36], and pre-treatment of MATB rat mammary cancer cells with recombinant human PAI-2 or slow infusion of the inhibitor with osmotic pumps led to a significant decrease in lung metastasis post intravenous (i.v) administration [37].

PAI-1 conjugated to A-chain cholera toxin as the cytotoxic agent or modified PAI-1 conjugated to saporin has also been used to target fibrosarcoma cells [38,39] with moderate cytotoxicity. However, PAI-2 has several distinct advantages over PAI-1 with regard to the lack of systemic or pathological consequences in sustained exposures and is 10,000 fold less active towards tPA which has a high affinity for fibrin [23]. PAI-2 is very stable in vitro compared to PAI-1 and

does not revert to a latent form in vitro or in vivo compared to PAI-1[23]. Furthermore, high blood levels of PAI-2 are unlikely to cause adverse health effects since the "abnormally" high PAI-2 levels found during late pregnancy (usually blood levels of PAI-2 are undetectable) are not associated with toxicity [23] and do not induce a human anti-mouse antibody response.

We have therefore investigated the use of PAI-2 as the basis of a new therapeutic agent. In this paper we report for the first time the new compound Bi-213-cDTPAa-PAI-2, ie alpha-PAI-2. SDS-PAGE experiments show that uPA inhibitory activity is maintained when the chelating agent cDTPAa is conjugated with PAI-2. The incorporation of ^{213}Bi into cDTPA-PAI2 was found to be efficient and the conjugate quite stable in serum, suggesting its usefulness in vivo. The short half-life of ^{213}Bi is adequate for labeling and the Ac-225 generator can be milked every two hours to obtain a practical source of Bi-213.

While MDA-MB-231 and MCF-7 have quite different uPA expressions, they have similar 37% survival values (i.e. ~ 2 μCi) when incubated with alpha-PAI-2. This implies that, since only a few alpha particle hits of the nucleus are required to kill a cell [1], compared with thousands for betas, even MCF-7 cells with low uPA levels can still receive a lethal dose at quite low levels of targeted activity. In addition, after incubation in 5 μCi of alpha-PAI-2, EGR-CMK significantly improved cell survival by a factor of 3.3 as a result of inhibition of the PAI-2 interaction with cellular uPA. Furthermore, cytotoxicity was not observed with freshly isolated normal human leukocytes incubated with alpha-PAI-2. Breast cancer cells incubated with alpha-BSA were also unaffected. Clearly, alpha-PAI-2 is very toxic to targeted cancer cells, whereas non-targeted cells are spared from the radiotoxicity arising from the alpha radiation.

CONCLUSIONS

We have combined the cytotoxicity of an alpha-emitting radioisotope (Bi-213) with the targeting potential of the uPA system to create a novel targeted therapy for breast cancer. This is the first report of targeted alpha cytotoxicity with alpha-PAI-2. The in vitro cytotoxicity of alpha-PAI-2 on breast cancer cells is shown to be highly specific. Neither PAI-2 nor the chelate cDTPA-PAI-2 is cytotoxic to breast cancer cells. Further, results for the non-specific control (alpha-BSA), and for alpha-PAI-2 incubated with normal human leukocytes, prove that the cell killing ability of alpha-PAI-2 depends critically on the targeting of cancer cells.

We have therefore validated, in vitro, the potential of alpha-PAI-2 as a novel therapeutic agent for breast cancer.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Coomassie stained 12% SDS-PAGE showing complex formation between high and low molecular weight tc-uPA and PAI-2 or cDTPA-PAI-2. Complexes were formed by incubating either labeled or unlabeled PAI-2 with equimolar amounts of tc-uPA for 1.5 h at 37°C. The reaction was terminated by adding 5-times non-reducing SDS-PAGE sample buffer (20% v/v) to the reaction mix. LMWt corresponds to low molecular weight uPA that retains enzymatic activity of native uPA.

Figure 2. PAI-2 inhibition of tc-uPA enzymatic activity. The uPA inhibitory activity of DTPA-PAI-2 (■) was compared to the inhibitory activity of unmodified PAI-2 (□). The assay was performed over a 5 minutes interval at 37°C in the presence of the Spectrozyme-UK substrate.

Figure 3. Analysis of ^{213}Bi labeled cDTPA-PAI-2 (alpha-PAI-2). (A) Aliquots (10 µl) of freshly eluted alpha-PAI-2 (filled bars) or ^{213}Bi (unfilled bars) were spotted onto the origin of separate ITLC strips. After mobilization, the strips were cut into 4 pieces for gamma counting. Fraction 1 corresponds to the sample origin while fraction 4 corresponds to the solvent front. (B) Serum stability of alpha-PAI-2 incubated at 37°C in human serum for 0 min (unfilled bars) and 45 min (filled bars) and subjected to ITLC and analysed as per (A).

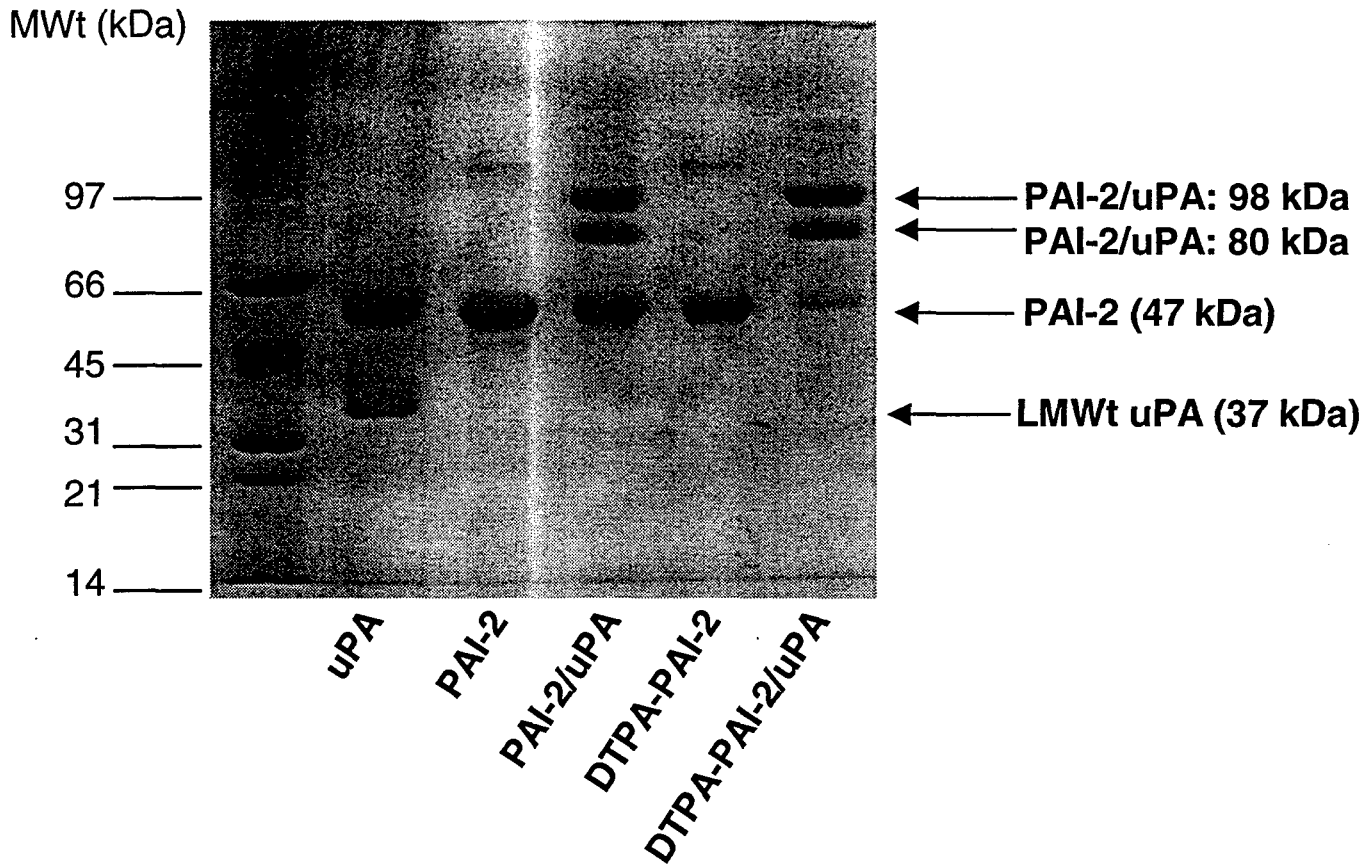
Figure 4. (A) Cell surface expression of endogenous uPA, uPAR and PAI-2. Viable MDA-MB-231 (filled bars) and MCF-7 (unfilled bars) cells were analysed for the specific expression of the various antigens by dual-colour flow cytometry as described in the methods. Values shown are means ± SD (n >3). (B) Immunohistochemistry of fixed, non-permeabilised MDA-MB-231 (panels I, II and III) and MCF-7 (panels IV, V, and VI) cells showing positively (brown staining) for uPA (II,V) and uPAR (III, VI) antigen. Panels I and IV represent isotype controls.

Figure 5. Representative cytotoxicity study of (A) MDA-MB-231 and (B) MCF-7 cells. Cells were either pre-incubated with 0.5 mM of the uPA specific inhibitor EGR-CMK (□) for 15 min at room temperature or with medium alone (■) after plasminogen treatment. Cells were then treated with varying concentrations of alpha-PAI-2 or a single concentration of alpha-BSA (▲), incubated overnight and cell survival measured and expressed as a percentage of cell survival of control cells. Controls consisted of RPMI medium alone. Values shown are the means of two experiments performed in triplicate.

Table 1 Percentage cell survival at 5μCi activity of alpha-proteins or at excess concentrations of cDTPA-PAI-2 and PAI-2 compared to controls

Cells	Alpha-PAI-2[1,2,4]		Alpha-BSA[1,3]		cDTPA-PAI-2[3]	PAI-2[3]
	-	+	-	+	(37.5 μg/mL)	(50 μg/mL)
MDA-MB-231	11.2 ± 2.9	39.8 ± 4.3	87.5 ± 5.4	87.3 ± 5.1	97.5 ± 2.1	98.0 ± 1.4
MCF-7	12.8 ± 2.6	39.5 ± 3.3	86.7 ± 4.8	85.8 ± 5.2	97.5 ± 1.3	97.5 ± 2.2
Leukocytes	98.0 ± 2.8	93.5 ± 6.4	ND		ND	ND

1 - and + indicate without and with EGR-CMK pre-treatment. ND = not determined.
2 Values shown are the means ± SD (n=6, each experiment performed in triplicate)
3 Values shown are the means ± SD (n=2, each experiment performed in triplicate)
4 Protein concentration of alpha-PAI-2 was approximately 2.5 μg/mL


MWt (kDa)
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66
45
31
21
14
uPA
PAI-2
PAI-2/uPA
DTPA-PAI-2
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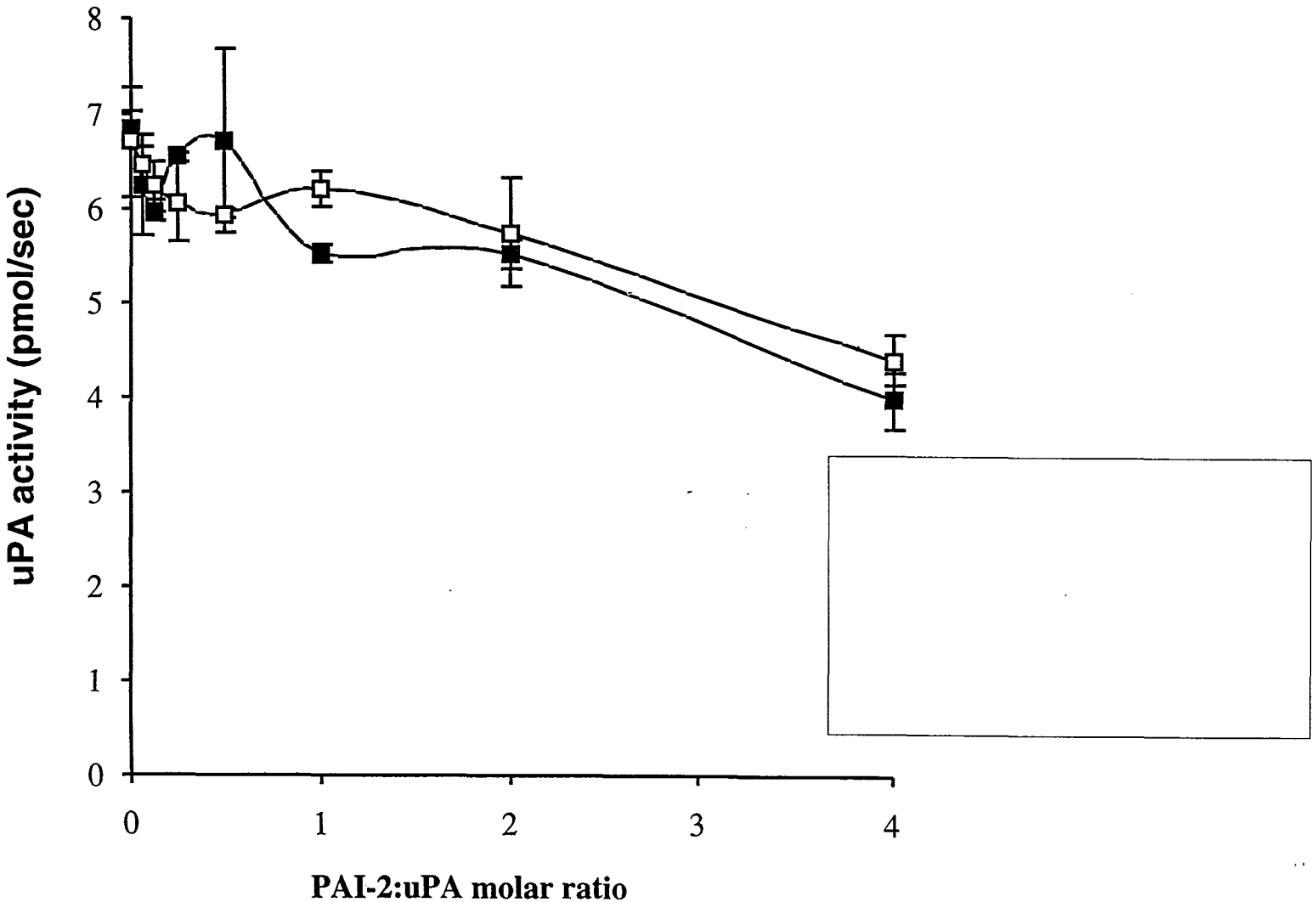
PAI-2 (47 kDa)
LMWt uPA (37 kDa)


uPA activity (pmol/sec)
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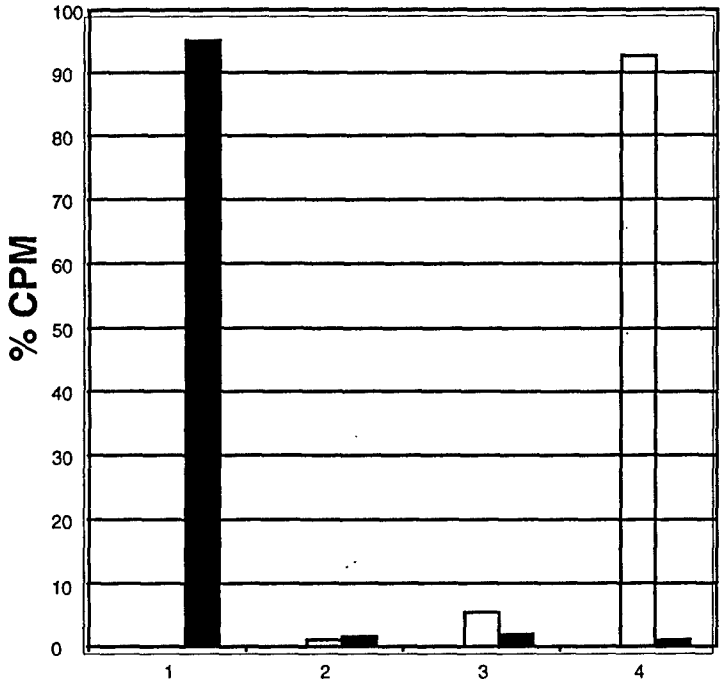
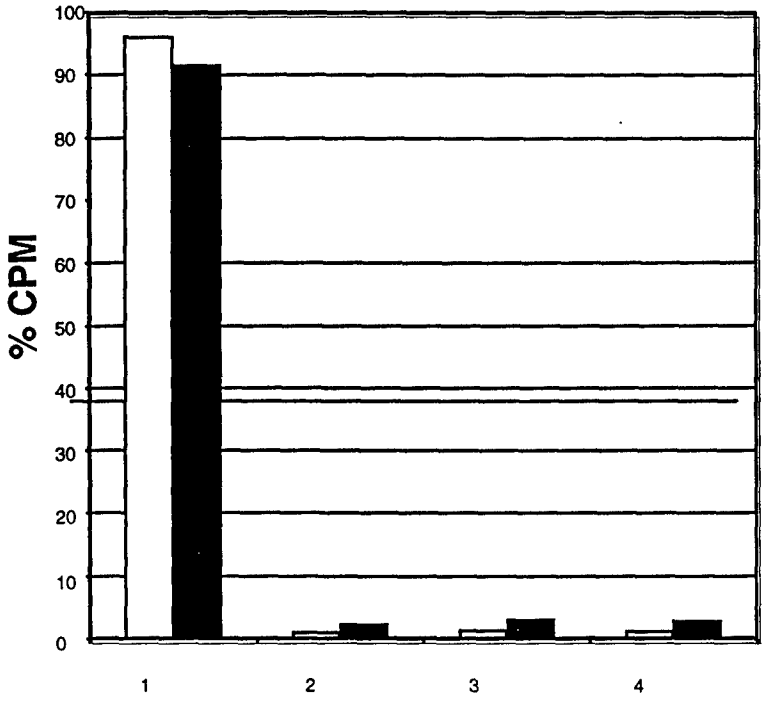
7
6
5
4
3
2
1
0
0
1
2
3
4
PAI-2:uPA molar ratio

A



Origin to solvent front fractions

B



Origin to solvent front fractions

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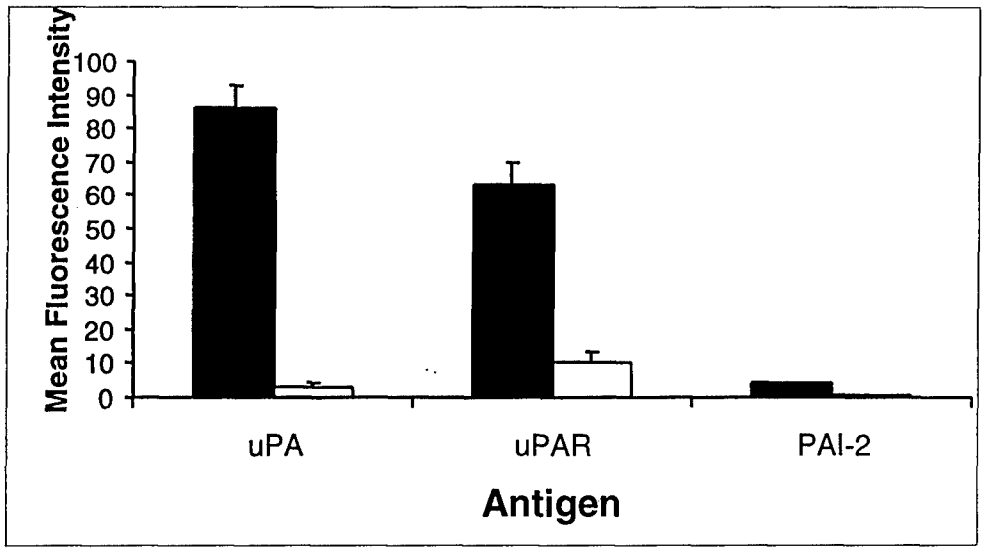
Mean Fluorescence Intensity
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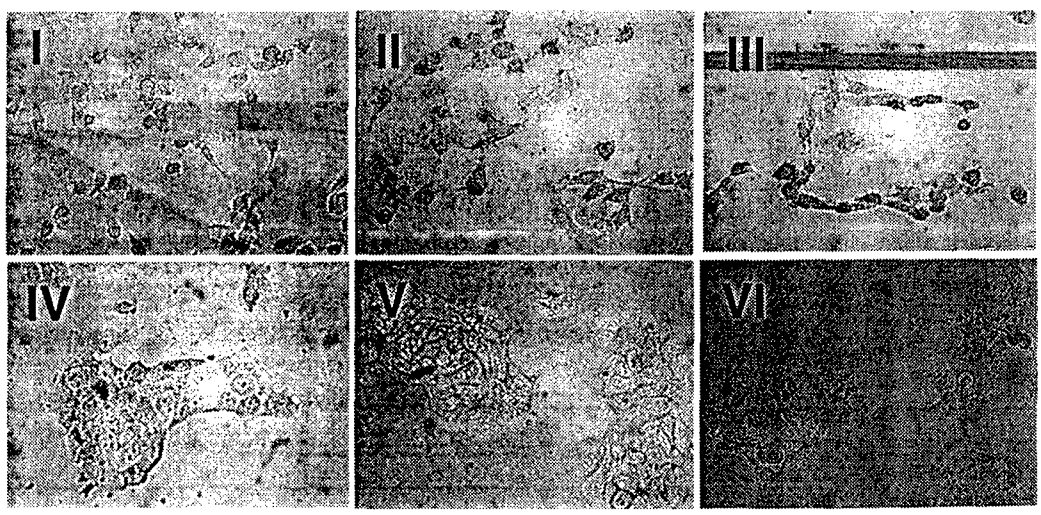
PAI-2
Antigen

B


I
II
III
IV
V
VI

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REPLY TO
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